Journal of Experimental Botany, Vol. 41, No. 233, pp. 1573–1578, December 1990

Effect of Nitrate on Peroxisome Ultrastructure and Catalase Activity in Nodules of *Lupinus albus* L. cv. Multolupa

C. LORENZO, M. M. LUCAS, A. VIVÓ and M. R. de FELIPE[1]

Unidad de Biología Vegetal, Instituto de Edafología y Biología Vegetal, C.S.I.C. Serrano, 115 dpdo. 28006 Madrid, Spain

Received 22 January 1990

ABSTRACT

This paper reports on an ultrastructural study of peroxisomes in infected nodule cells of *Lupinus albus* L. cv. Multolupa plants grown with and without nitrate (in short-term experiments). Cytochemical localization of catalase and peroxidase was performed applying the diamino-benzidine (DAB) technique in these tissues.

The infected cells presented a mean of seven peroxisomes, the maximum being 16 in some cells. Peroxisome shapes proved to be fairly round or egg-shaped, with maximum and minimum diameter means of 0·35 and 0·18 μm, respectively. They were preferentially positioned on the cell periphery. The intense osmiophilic staining obtained by applying the DAB technique indicates a strong catalase activity reaction in these microbodies.

The addition of nitrate (20 mol m^{-3}) to the growing plants exerted a negative effect on nitrogenase activity, which diminished by 31·6% after 5 d of treatment. Severe alterations in the ultrastructure of microbodies, bacteroids, and peribacteroidal membranes were observed.

Cytochemical data show a reduction in catalase localization in peroxisomes and an increased peroxidase activity in the cytosol.

Finally, leghaemoglobin (Lb) localization was studied in nitrate-grown plants, confirming our previous observation of a decrease in this protein. Discussion focuses on the involvement of these results in tissue senescence of the nodules following nitrate application.

Key words: Peroxisomes, diamino-benzidine, nitrate, catalase, leghaemoglobin.

INTRODUCTION

Senescence processes have been traditionally associated with variations in the enzyme levels which protect the tissues from toxic oxygen species (H_2O_2, O_2^-, $\cdot HO$). Among these enzymes there are superoxide dismutase (SOD, EC. 1.15.1.1), catalase (EC. 1.11.1.6) and peroxidase (EC. 1.11.1.7). Becana, Aparicio-Tejo, and Sánchez-Díaz (1988) obtained indirect evidence of the accumulation of H_2O_2 during legume nodule senescence. The same authors also found a catalase decrease in the cytoplasm, induced by NO_3^- application to the plants. Catalase levels and nitrogen fixation have been shown to be positively correlated (Francis and Alexander, 1972).

One of the nodule components highly sensitive to these toxic oxygen species is leghaemoglobin (Lb), since oxidation can affect its physiological function. Puppo and Halliwell (1988) observed that, under '*in vitro*' conditions, H_2O_2 oxidizes ferroleghaemoglobin to leghaemoglobin (IV), triggering the release of superoxide radical (O_2^-). Likewise, Puppo, Dimitrijevic, and Rigaud (1982) proved that NO_2^- derived from NO_3^- reduction oxidizes the ferroleghaemoglobin to non-functional ferrileghaemoglobin.

On the other hand, it is only recently that attention has been paid to the role played by peroxisomes in the metabolism of legume nodules. In soybean and cowpea plants, which transport the fixed nitrogen in the form of ureides, peroxisomes in the interstitial cells have been studied (Newcomb, Tandon, and Kowal, 1985*b*; Selker and Newcomb, 1985; Webb and Newcomb, 1987). In these peroxisomes, which are larger in size than those in the infected cells, the final steps of ureide metabolism take place (Hanks, Schubert, and Tolbert, 1983). In

[1] To whom correspondence should be addressed.

legumes which transport nitrogen in the form of amides, such as alfalfa, vetch, and pea, these large peroxisomes do not exist in the uninfected cells of their nodules (Newcomb, Selker, Tandon, Meng, and Kowal, 1985*a*).

Lupins are amide transporters (Bergersen, 1980, 1982) and are characterized by the fact that the central part of the nodule consists exclusively of infected cells. The structure, morphology, position, etc. of peroxisomes in the infected cells in *Lupinus* nodules are unknown. Moreover, the histochemical localization of the enzymes catalase and peroxidase in the infected nodule cells has not yet been reported, although it is well-known for the uninfected cells (Marks and Sprent, 1974; Newcomb *et al.*, 1985*b*; Vaughn, 1985; Kaneko and Newcomb, 1987).

This research pursues the following aims: (1) a structural study of peroxisomes in *Lupinus albus* L. cv. Multolupa as well as catalase localization therein; and (2) to examine the effect of nitrate on the localization of enzyme activity of catalase and peroxidase, and on leghaemoglobin distribution.

MATERIALS AND METHODS

Plant material

Sterile germinated seeds of *Lupinus albus* L. cv. Multolupa were planted (three seedling per jar) in autoclaved Leonard jars filled with vermiculite and sand (2:1 v/v), and inoculated with *Bradyrhizobium* sp. *(Lupinus)* strain ISLU 16 (from San José de La Rinconada, Agronomy Research Service, Sevilla, Spain). The inoculum contained 10^9 bacteria cm^{-3}. After 1 week, the water in the reservoir in the Leonard assembly was replaced by the appropriate nutrient solution and about 1 week later the plants were selected to one per pot. The growth chamber conditions were 16/8 h light/dark cycle, at 25 °C, 70% RH and irradiance level of 190 $\mu E\ m^{-2}\ s^{-1}$ supplied by fluorescent tubes (Silvania Gro-Lux). Potassium nitrate (20 $cm^3\ m^{-3}$) was added to well-nodulated plants 40 d after inoculation. The amount of potassium in the controls was balanced by K_2SO_4 and KCl.

Plants were harvested 5 d and 10 d after NO_3^- addition to the nutrient solution. Harvesting was initiated 4 h after commencement of the light period.

Acetylene reduction assay

Nitrogen fixation measured as acetylene reduction assay (ARA) was performed on nodulated roots enclosed in 100 cm^3 tubes fitted with rubber stoppers (de Felipe, Fernandez-Pascual, and Pozuelo, 1987). Gas samples were taken after 1 h and analysed for ethylene and acetylene in a Perkin Elmer 8310 gas chromatograph equipped with a hydrogen flame ionization detector and a column filled with Porapak R using nitrogen as carrier gas at a flow rate of 50 $cm^3\ min^{-1}$.

Electron microscopy and cytochemistry

Nodules were detached from the roots and selected for a comparable stage of development (main root nodules with similar aspects). Small samples of nodule tissue were fixed in 2·5% glutaraldehyde in 50 mol m^{-3} Na-cacodylate buffer (pH 7·4) and vacuum infiltrated. Some of the samples were postfixed in 1% OsO_4 (w/v) in the same buffer at 2 °C and processed for conventional electron microscopy.

The cytochemical localization of catalase and peroxidase was carried out according to the method of Mueller and Beckman (1978) modified by de Felipe, Lucas, and Pozuelo (1988). Nodule samples of 3·0 mm in length were collected in 50 mol m^{-3} K-phosphate buffer (pH 6·8) and immediately fixed in 2·5% glutaraldehyde in the same buffer for 2 h. Following fixation, the samples were washed (4 × 5 min) and pre-incubated for 1 h in the dark at room temperature in the following solution: 0·2% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, Mo., USA) in 50 mol m^{-3} 2-amino-2-methyl-1,3-propanediol (AMPD, Sigma) buffer, pH 9·0. Incubation was performed for 1 h in the dark at 37 °C in the above medium plus 0·02% H_2O_2 (v/v).

To distinguish peroxidase from catalase DAB reaction, 50 mol m^{-3} 3-amino 1,2,4-triazol (AT) was used as a catalase inhibitor. Control test minus H_2O_2 or plus 10 mol m^{-3} KCN in both the pre-incubation and the incubation mediums were also carried out.

Post-incubation rinses were performed with 50 mol m^{-3} AMPD, pH 9·0 followed by 50 mol m^{-3} K-phosphate buffer for 30 min. The samples were then processed for electron microscopy and finally embedded in Araldite (Durcupan ACM).

Ultra-thin sections were cut with a diamond knife fitted to a Reichert ultramicrotome, post-stained with lead citrate (Reynolds, 1963) and examined with a Philips 300 electron microscope at 80 kV.

Morphometric parameters were measured using an Image Analyser (Kontron MOP Videoplan).

Leghaemoglobin localization

Unfixed sections in OsO_4 were collected on uncoated nickelgrids. Lb localization was achieved as described in Vivo, Andreu, de la Viña, and de Felipe (1989). The goat anti-rabbit IgG-Au used was 15 nm diameter colloidal gold (Janssen, Life Sci.).

RESULTS

Peroxisome ultrastructure in Lupinus *nodule*

Peroxisomes are fairly scarce in the nodules of plants which transport fixed nitrogen in the form of amides (Vaughn, Duke, and Henson, 1982). In fact this was the authors' impression when initiating the structural study of *Lupinus* nodules. By applying the DAB reaction, however, we were able to observe up to 16 peroxisomes in an infected cell and to determine their morphometric characteristics and distribution.

Morphological characteristics: The peroxisomes observed (Table 1) present average diameters of 0·35 μm (maximum) and 0·18 μm (minimum). Their shapes can be round, egg-shaped or, as occurred in a few cases, triangular. They do not show any paracrystalline inclusions nor any detectable fibrillar content.

Distribution: The peroxisomes are preferentially located

TABLE 1. *Morphometric parameters of infected cell peroxisomes in nodules of control plants of* Lupinus albus *L. cv. Multolupa*

Area	μm^2	0·09 ± 0·03
Perimeter	μm^2	1·07 ± 0·23
Maximum diameter	μm^2	0·35 ± 0·09
Minimum diameter	μm^2	0·18 ± 0·05

on the periphery of the infected cells. This may be due to the displacement undergone by the organelles of these cells as a consequence of the considerable mass of bacteroids in the cell centre. They are often located in the vicinity of rough endoplasmic reticulum (RER) or even in close association with it (Plate 1A). Occasionally groupings of two or three peroxisomes can be observed.

Catalase localization in Lupinus albus *L. cv. Multolupa nodules*

The peroxisomes of the infected cells showed a positive reaction to DAB with intense blackening of the inside of the organelles (Plates 1B, C). In the bacteroids the appearance of small osmiophilic precipitates was noted at different sites, probably due to catalase activity (Plate ID). Likewise, ribosome darkening was observed, both in ER-association and in the cytosol.

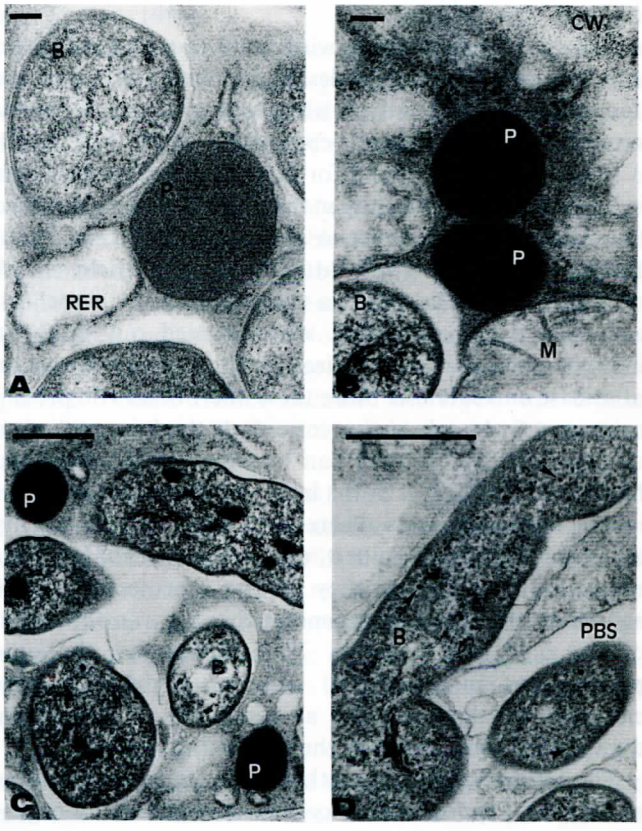

PLATE. 1. Electron micrographs of *Lupinus albus* L. cv. Multolupa plants grown without nitrate (controls). (A) Infected cell from a 45-d-old root nodule. The single peroxisomal membrane can be easily observed. Rough endoplasmic reticulum is closely associated to the peroxisome. ×88 500. Bar = 0·1 μm. (B) Infected cell from a 50-d-old root nodule after performing the DAB technique. The two peroxisomes show intense darkening. The reaction product is evenly distributed throughout the peroxisomal matrix. ×88 500. Bar = 0·1 μm. (C) Infected cell from a 50-d-old nodule showing DAB-marked peroxisomes and bacteroids. ×46 500. Bar = 0·5 μm. (D) Infected cell from a 50-d-old nodule. Bacteroids show a small osmiophilic precipitate (arrows) which could be related to catalase activity. ×72 500. Bar = 0·5 μm. Key to lettering: B, bacteroid; CW, cell wall; M, mitochondria; P, peroxisome; PBS, peribacteroidal space; RER, rough endoplasmic reticulum.

Regarding the controls used to inhibit enzyme activity, H_2O_2 exclusion from the incubation medium did not entail a significant reduction of the reaction. The use of 10 mol m^{-3} KCN, however, led to a considerable reaction loss in all structures. AT did not achieve complete catalase inhibition, as reaction residues were observed in the peroxisomes (not shown).

Combined nitrogen effect

Effect on ultrastructure and nitrogenase activity: The application of combined nitrogen (20 mol m^{-3}) to nodulated plants gave rise to a general structural degradation of the nodule, causing tissue alterations in the cytoplasm as well as in the bacteroids. Application of the 20 mol m^{-3} dose for 10 d caused the peribacteroidal spaces to become wider and the peribacteroidal membranes to become sinuous. These membranes are also broken at certain sites. The bacteroids adopt irregular shapes within the peribacteroidal spaces and eventually come to consist solely of their membranes. With regard to the effect on the cytoplasm, intense vesiculation was observed with the consequential diminishing of the subcellular organelles. A few exceptional peroxisomes were found, but with altered structure and ruptured membranes.

The effect of nitrate on nitrogenase activity can be seen in Table 2. The enzyme inhibition rate amounts to 31·6% and 69·7% 5 d and 10 d after NO_3^- administration, respectively. The drop in nitrogenase activity in control plants is due to nodule age, since maximum activity was achieved with 40-d-old nodules (24·7 μmol C_2H_4 h^{-1} g^{-1} nodule fresh weight), and then declined.

Effect on catalase and peroxidase localization: With respect to catalase localization, an almost total disappearance of the DAB reaction was observed in the peroxisomes in nitrate-treated plants. These peroxisomes showed only slight darkening, ruptured membranes and diffuse contours (Plate 2A, B). In the bacteroids the DAB reaction was evident by the presence of a small black precipitate on the cytosol, with no remarkable difference being noticed when compared with control plants.

TABLE 2. *Effect of nitrate (20 mol m^{-3}) on nitrogenase activity and shoot fresh weight in plants of* Lupinus albus *L. cv. Multolupa*

Values of the Acetylene Reduction Assay (ARA) are expressed as μmol C_2H_4 h^{-1} g^{-1} nodule fresh weight. Values of shoot fresh weight are given in g. Each value is the mean ± s.d. of 12 plants.

		Days of treatment with 20 mol m^{-3} NO_3^-	
		5 d	10 d
ARA	Control plants	16·5 ± 6·3	5·3 ± 2·8
	NO_3^- grown plants	10·7 ± 1·5	1·6 ± 0·3
Inhibition of nitrogenase %		31·6	69·7
Shoot fresh weight	Control plants	17·1 ± 3·8	23·4 ± 5·5
	NO_3^--grown plants	18·2 ± 1·4	20·2 ± 2·8

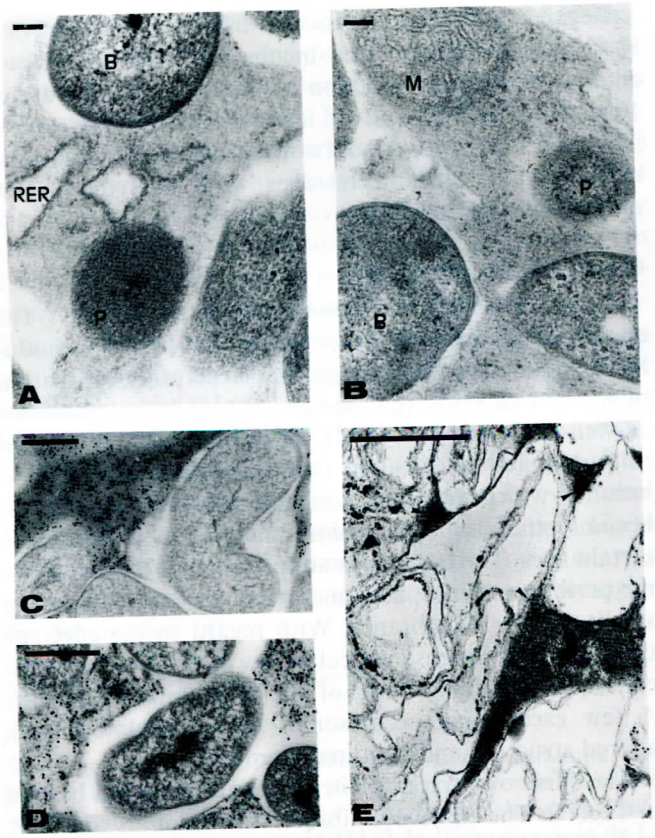

PLATE. 2. Effect of nitrate (20 mol m^{-3}) on peroxisome ultrastructure, catalase activity, and leghaemoglobin localization in *Lupinus albus* L. cv. Multolupa root nodules. (A) Electron micrograph of an infected cell from a 45-d-old nodule after employment of the DAB technique. The plant was exposed to nitrate for 5 d. The peroxisomal membrane is lost at certain sites but the peroxisome shape is fairly regular. The darkening is not as intense as in control plants. There is a more intense reaction in the centre of the organelle. ×88 500. Bar = 0·1 μm. (B) Electron micrograph of an infected cell from a 50-d-old nodule. After 10 d of exposure to nitrate, there is only a slight ring-shaped reaction and the peroxisomal membrane is lost. Bacteroids also show some small osmiophilic precipitates. Peribacteroidal membranes are damaged or lost. ×88 500. Bar = 0·1 μm. (C) Immunogold localization of leghaemoglobin in a 50-d-old nodule from a control plant. ×32 000. Bar = 0·5 μm. (D) Immunogold localization of leghaemoglobin in a 50-d-old nodule after 10 d of exposure to nitrate. ×42 000. Bar = 0·5 μm. (E) Electron micrograph showing the appearance of some infected cells and leghaemoglobin localization after 10 d of exposure to nitrate. ×65 000. Bar = 0·5 μm.

An increase in blackening of the cytosol of the infected cells was observed following the DAB reaction. This could be related to peroxidase activity present in the cytosol of nodular cells, or else to residual catalase or pseudo-peroxidase activity of catalase resulting from the rupture of peroxisomal membranes.

Effect on the immunocytochemical location of leghaemoglobin: Lb localization in the infected cells of nodules grown with 20 mol m^{-3} NO_3^- followed the same localization model as in the control plants, and proved especially relevant in the cytosol of the infected cells (Plate 2C, D). The label was, however, much less dense. Density was determined by counting the number of particles per μm^2 and yielded a ratio of 9:2 for controls and 20 mol m^{-3} NO_3^- after 10 d of plant growth in KNO_3.

Leghaemoglobin localization remains in the cytosolic areas even in cells showing a highly advanced stage of senescence (Plate 2E).

DISCUSSION

Structural and cytochemical studies of nodular cell peroxisomes determining their enzyme activities have been conducted by several authors on soybean plants (Newcomb *et al.*, 1985*b*; Vaughn, 1985; Vaughn and Stegink, 1987). In soybean nodules peroxisomes play an important role, as they constitute the site where ureide metabolism takes place prior to its transfer to the aerial part.

In lupin plants, the fixed nitrogen is transported in the form of amides, essentially as asparagine (Bergersen, 1982), which may suggest that in these nodules the peroxisomes do not play such an essential role as in soybean type nodules.

The cytochemical study with DAB in nodular *Lupinus* infected cells (these nodules lack interstitial cells) has demonstrated the relatively abundant existence of peroxisomes. The size of these peroxisomes (0·18–0·35 μm Ø) is smaller than that found for interstitial cell peroxisomes in soybean plants, but larger than the diameters recorded for microbodies on soybean nodule infected cells (Newcomb *et al.*, 1985*b*). These latter peroxisomes yield a weak catalase reaction, as well as a weak reaction for cytochemical uricase determination, when carried out using an indirect method which requires consecutive enzyme action of uricase and catalase. Since these microbodies do present uricase activity when the cerium method is applied, a minimal catalase activity can be expected (Kaneko and Newcomb, 1987). It is in the large specialized peroxisomes in the interstitial cells where the major uricase activity takes place (Hanks, Tolbert, and Schubert., 1981; Hanks *et al.*, 1983) and hence they require a protection device against the H_2O_2 which is generated in the transformation of uric acid into allantoin. This implies that the large peroxisomes in uninfected cells of soybean nodules enhanced catalase activity, as compared to that of the diminutive microbodies in the infected cells.

The intense catalase reaction obtained in peroxisomes of infected *Lupinus* nodule cell characterizes these organelles as the main site of nodular catalase activity. This fact, together with the small peroxisome size and their uniform appearance, as well as the amorphous matrix, suggest that they are non-specialized peroxisomes and their most likely function is H_2O_2 detoxification.

Goldman and Blobel (1978) state that catalase, as with other peroxisomal enzymes, is synthesized in the cytosol-free polysomes and post-transcriptionally transported within the peroxisome. We are able to observe deposits of the reaction product on ribosomes in the cytosol, as well as on RER-associated ribosomes.

With respect to the existence of catalase activity in the bacteroids, some authors have detected its presence in white clover and soybean bacteroids (Francis and Alexander, 1972) as well as in alfalfa (Becana *et al.*, 1988). In our study we obtained small osmiophilic precipitates in the cytosol of the bacteroids which can be assumed to relate to catalase activity.

The effect of combined nitrogen

It is a well-known fact that combined nitrogen application in legumes with effective and firmly established nodulation entails a drop in nitrogenase activity. The causes underlying this inhibition of the N-fixation mechanism have not yet been satisfactorily clarified, although it seems to be generally accepted that in the initial stages of NO_3^- exposure, the drop in nitrogenase may be due to the energy deficit suffered by the bacteroids when the O_2 diffusion barrier of the nodule is increased (Becana and Sprent, 1987).

With longer exposure times and/or higher NO_3^- concentrations, nitrate eventually penetrates the infected zone of the nodule, where it is reduced to NO_2^- by the nitrate reductases of the plant (EC. 1.6.6.1) and the bacteroid (EC. 1.7.99.4).

Nitrite combines reversibly with ferro-Lb and oxidizes it to ferri-Lb, a form unable to carry O_2. In addition, oxygenated ferro-Lb (O_2-Lb) is highly sensitive to self-oxidation, and this process results in the release of superoxide radical (O_2^-). Nodular superoxide dismutase would act upon O_2^-, rendering H_2O_2 and O_2. Catalase protects nodule tissues because: (i): H_2O_2 can oxidize ferro-Lb, and (ii) H_2O_2 can combine with O_2^- to form the highly oxidant ·HO.

Becana *et al.* (1988) found that application of 20 mol m^{-3} NO_3^- to nodulated alfalfa plants led to a 20% decrease in catalase activity in the cytosol, whereas in the bacteroids catalase activity remained practically constant. This result appears to be consistent with our own cytochemical findings, since we observed a catalase activity decrease in the peroxisomes of plants treated with nitrate (20 mol m^{-3}).

In addition, all nodular components in the infected cells showed nitrate-induced morphological deterioration, and this fact was even clearer in the case of peroxisomes. This damage could be due to impairment of the nodule defence mechanisms against toxic species, triggering accelerated senescence of the nodular tissue (Pladys, Van de Sype, Gleyzal, and Rigaud, 1989).

The drop in catalase localization ran parallel to a considerable loss of '*in situ*' Lb localization. The former implies a lower level of defences in nodule tissues, and the latter implies that bacteroid respiration is hindered. These two points, together with the observed drop in nitrogenase activity, bring us to conclude that nitrate feeding has initiated a senescence process.

ACKNOWLEDGEMENTS

The financial support of the CICYT (Spain, PB 88-0013) is greatly acknowledged. The authors wish to thank Mrs Carmen de Mesa and María Luísa Melendo for competent and skilful technical assistance, and Mr Fernando Pinto from the Electron Microscope Laboratory for facilities.

LITERATURE CITED

Becana, M., Aparicio-Tejo, P., and Sanchez-Diaz, M., 1988. Nitrate and hydrogen peroxide metabolism in *Medicago sativa* nodules and possible effect on leghaemoglobin function. *Physiologia plantarum*, **38,** 65–9.

—— and Sprent, J. I., 1987. Nitrogen fixation and nitrate reduction in the root nodules of legumes. Ibid. **70,** 757–65.

Bergersen, F. J., 1980. Leghaemoglobin, oxygen supply and nitrogen fixation: studies with soybean nodules. In *Nitrogen fixation*. Eds W. D. P. Stewart and J. R. Gallon. Academic Press. New York. London. Pp. 160.

—— 1982. *Root nodules of legumes: structure and functions.* Research Studies Press. England.

de Felipe, M. R., Fernandez-Pascual, M., and Pozuelo, J. M., 1987. Effects of herbicides Lindex and Simazine on chloroplast and nodule development, nodule activity, and grain yield in *Lupinus albus* L. *Plant and Soil*, **101,** 99–105.

—— Lucas, M. M., and Pozuelo, J. M., 1988. Cytochemical study of catalase and peroxidase in the mesophyll of *Lolium rigidum* plants treated with isoproturon. *Journal of Plant Physiology*, **132,** 67–73.

Francis, A. J., and Alexander, M., 1972. Catalase activity and nitrogen fixation in legume root nodules. *Canadian Journal of Microbiology*, **18,** 861–4.

Goldman, B. M., and Blobel, G., 1978. Biogenesis of peroxisomes: intracellular site of synthesis of catalase and uricase. *Proceedings of the National Academy of Science of USA*, **75,** 5066–70.

Hanks, J. F., Schubert, K. R., and Tolbert, N. E., 1983. Isolation and characteristics of infected and uninfected cells from soybean nodules. Role of uninfected cells in ureide production. *Plant Physiology*, **71,** 869–73.

—— Tolbert, N. W., and Schubert, K. R., 1981. Localization of enzymes of ureide biosynthesis in peroxisomes and microsomes of nodules. Ibid. **68,** 65–9.

Kaneko, Y., and Newcomb, E. H., 1987. Cytochemical localization of uricase and catalase in developing root nodules of soybean. *Protoplasma*, **140,** 1–12.

Marks, I., and Sprent, J. I., 1974. The localization of enzymes in fixed sections of soybean root nodules by electron microscopy. *Journal of Cell Science*, **16,** 623–37.

Mueller, W. C., and Beckman, C. H., 1978. Ultrastructural localization of polyphenoloxidase and peroxidase in roots and hypocotyls of cotton seedlings. *Canadian Journal of Botany*, **56,** 1578–87.

Newcomb, E. H., Selker, J. M. L., Tandon, Sh, R., Meng, F., and Kowal, R. R., 1985*a*. Uninfected cells in ureide- and amide- exporting legume root nodules. In *Nitrogen fixation and CO_2 metabolism*. Eds P. W. Ludden and J. E. Burris. Elsevier Science, New York. Pp. 831–40.

—— Tandon, Sh, R., and Kowal, R. R., 1985*b*. Ultrastructural specialization for ureide production in uninfected cells of soybean root nodules. *Protoplasma*, **125,** 1–12.

Pladys, D., Van de Sype, G., Gleyzal, C., and Rigaud, J., 1989. Sur l'effet du faibles concentrations de nitrate sur

l'ultrastructure du tissu central de nodosites de *Phaseolus vulgaris* L. *Comptes Rendues Academie de Science de Paris*, **308,** 261–8.

PUPPO, A., DIMITRIJEVIC L., and RIGAUD, J., 1982. Possible involvement of nodule superoxide dismutase and catalase in leghaemoglobin protection. *Planta*, **156,** 374–9.

—— and HALLIWELL, B., 1988. Generation of hydroxyl radicals by soybean nodule leghaemoglobin. Ibid. **173,** 405–10.

REYNOLDS, E. S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology*, **17,** 208–13.

SELKER, J. M. L., and NEWCOMB, E. H., 1985. Spatial relationships between uninfected and infected cells in root nodules of soybean. *Planta*, **165,** 446–54.

VAUGHN, K. C., 1985. Structural and cytochemical localization of three specialized peroxisome types in soybean. *Physiologia plantarum*, **64,** 1–12.

—— DUKE, S. H., and HENSON, C. A., 1982. Ultrastructural localization of urate oxidase in nodules of *Sesbania exaltata*, *Glycine max*, and *Medicago sativa*. *Histochemistry*, **74,** 309–18.

—— and STEGINK, S. J., 1987. Peroxisomes of root nodule vascular parenchyma cells contain a 'nodule-specific' urate oxidase. *Physiologia plantarum*, **71,** 251–6.

VIVO, A., ANDREU, J. M., DE LA VINA, S., and DE FELIPE, M. R., 1989. Leghaemoglobin in lupin plants (*Lupinus albus* cv. Multolupa). *Plant Physiology*, **90,** 452–7.

WEBB, M. A., and NEWCOMB, E. H., 1987. Cellular compartmentation of ureide biogenesis in root nodules of cowpea (*Vigna unguiculata* (L.) Walp.) *Planta*, **172,** 162–75.